SPANDIDOS PUBLICATIONS

MOLECULAR AND CLINICAL ONCOLOGY 1: 575-581, 2013

Target therapy in NSCLC patients: Relevant clinical agents and tumour molecular characterisation (Review)

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Received October 26, 2012; Accepted February 26, 2013

DOI: 10.3892/mco.2013.100

Abstract. In recent years, a number of new agents that target specific molecular pathways in non-small cell lung cancer (NSCLC) have been investigated. Much effort has been focused on identifying specific markers that are predictive of treatment response, given that a tailored approach would maximise the therapeutic index and cost-effectiveness. Gefitinib and erlotinib are selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR-TKIs) and have produced good results in selected cases in terms of objective response rate and overall survival. At present, *EGFR* gene mutations are considered the most important predictors of clinical response to TKI therapy and tumour characterisation for these alterations is mandatory prior to any decision making. *Echinoderm microtubule-like protein 4-anaplastic lymphoma kinase* (*EML4-ALK)* translocation is another alteration capable of predicting the efficacy of anti-ALK agents, such as crizotinib. Moreover, emerging target agents, such as MET inhibitors, are likely to increase the amount of molecular characterisation required before a decision is made on treatment. The main limiting factor for adequate characterisation of metastatic NSCLC patients is the small quantity of tumour cells available for molecular analysis. In this study, we provided an overview of the most important and clinically relevant target agents in NSCLC patients as well as the most important mechanisms of resistance. The issue of the scant amount of biological samples available for analysis as well as alternative sampling approaches such as plasma- or serum-derived DNA were also examined.

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Key words: non-small cell lung cancer, epidermal growth factor receptor tyrosine kinase inhibitors, echinoderm microtubule-like protein 4-anaplastic lymphoma kinase, MET, plasma/serum

Contents

1. Introduction

Personalised treatment of cancer patients has become a reality in the last few years, with many drugs having been developed that target specific altered pathways. Concerning lung cancer, gefitinib and erlotinib are the first drugs to have demonstrated a good response in the treatment of patients with specific alterations in the *EGFR* gene (1,2) rendering molecular characterisation of the tumour crucial prior to taking any decisions regarding therapy. Other drugs, such as crizotinib, have demonstrated that they produce a high response percentage in patients carrying the *Echinoderm microtubule-like protein 4-anaplastic lymphoma kinase* (*EML4-ALK)* translocation (3) and, more recently, in patients carrying the *ROS1* rearrangement (4) or *MET* amplification (5). Other target agents, such as MET inhibitors, are used in clinical trials, with the emergence of very promising results (6,7).

The potential of utilizing molecular-targeted agents promotes analysis of the tumour for molecular alterations, in order to identify the best and most effective clinical treatment.

As lung cancer is diagnosed principally at advanced stages, the only biological material available for molecular analyses is derived from biopsy or needle aspirates, and is consequently lacking in quantity. Various technical improvements have been investigated in an attempt to obtain a greater amount of tumour cells.

In this review, the principal target agents that play a role in clinical practice, and the issues associated with the scarcity of tumour material required for molecular analyses were examined.

2. Clinically-relevant target drugs

EGFR-TKIs. Inhibition of the EGFR pathway with tyrosine kinase inhibitors (TKIs) has proven to be an effective treatment

strategy for advanced non-small cell lung cancer (NSCLC) (8-10). TKIs are a class of drugs that act on the EGFR ATP-binding site, leading to the reversible blocking of downstream signalling pathway activation. In 2004, three different research groups showed that EGFR-TK domain mutations are associated with the response of NSCLC patients to gefitinib (11-13) or erlotinib (13). Somatic mutations are more frequently observed in patients with clinical features known to be associated with TKI sensitivity, such as female, adenocarcinoma histology, Asian ethnicity and non-smoking history. Following these initial observations, the majority of *EGFR* mutations have been reported to be found in the first four TK domain exons (14-19).

The most common *EGFR*-sensitising mutations, accounting for 85-90% of all those found in NSCLC, include exon 19 deletion (loss of codons 746-750, ELREA amino acid sequence) and exon 21 L858R substitution. The two mutations have been shown to enhance EGFR kinase activity and activate its downstream signalling, playing a pivotal role in NSCLC cell survival (12,20). EGFR-TKIs are thought to neutralise the excessive survival signals to which cancer cells are 'addicted', leading to marked apoptosis (20,21). Moreover, activating *EGFR* mutations have also been shown to enhance gefitinib affinity by increasing its activity (22). Point mutations in exon 18 (G719A/C) occur in ~5% of cases, which are associated with oncogenic potential in both cell culture and transgenic mouse studies (14,18,23) and are also correlated with moderate TKI sensitivity (23,24). A large number of studies has reported a significantly higher response rate (ORR >80%), OS and TTP in patients with activating EGFR mutations compared to the wild-type individuals (ORR <10%) (25-35).

In view of the results reported by the IPASS study (1), gefitinib (IRESSA, AstraZeneca Pharmaceuticals, Wilmington, DE, USA) was the first TKI approved by the European Medicines Agency (EMEA) for all lines of therapy in adults with locally advanced or metastatic NSCLC with activating EGFR tyrosine kinase mutations. Results of the EURTAC study (2) have led to the approval of Erlotinib (TARCEVA Genentech, Inc., South San Francisco, CA, and OSI Pharmaceuticals, Inc., Melville, NY, USA), already approved for the treatment of locally advanced or metastatic NSCLC following the failure of at least one prior chemotherapy regimen, for the first-line treatment of same stage patients carrying an *EGFR* mutation. Results of the phase II LUX-LUNG 2 study have demonstrated that afatinib, an irreversible ErbB family blocker of EGFR, HER2 and HER4, showed significant activity in *EGFR* mutated patients (36). Moreover, preliminary results from the randomised phase III study LUX-LUNG 3 have demonstrated that afatinib significantly prolonged PFS compared to pemetrexed/cisplatinum treatment (37).

Although EGFR-TKI treatment shows good response rates and PFS in NSCLC patients with *EGFR* gene mutations, acquired resistance to treatment almost always develops after a median time of approximately 10 months from the initiation of treatment. Different genotypic and histological mechanisms of resistance have therefore been suggested (38).

Approximately half of cancers that acquired resistance to EGFR-TKIs developed a secondary mutation in the EGFR kinase domain involving methionine to threonine substitution in codon 790 (T790M) of exon 20 (39-41). This mutation is acquired through selective pressure during treatment, as it is rarely detected in tumours in untreated patients (40).

It has been demonstrated that T790M mutated cells show a growth disadvantage compared to wild-type cells, and these differential growth kinetics may be partly responsible for the 'flare' and 're-response' phenomenon observed in some patients with acquired response. Following the withdrawal of the selective pressure with a TKI, previously arrested TKI-sensitive cells can repopulate more rapidly compared with resistance cells and tumours may regain sensitivity to TKI (42). Additionally, in patients with acquired resistance, T790M has been found to be associated with a more indolent phenotype (43). Other less common mutations conferring modest resistance to EGFR-TKIs include the D761Y substitution and insertions in exon 20 (44,45). The favourable prognosis associated with the presence of T790M on re-biopsy suggests that re-biopsies play an important clinical role in the management of these patients (42,46).

As the acquisition of T790M reduces the efficacy of ATP-competitive inhibitors, one strategy for preventing or overcoming EGFR-TKI resistance may be the use of agents that bind and inhibit EGFR through a distinct, non-ATP competitive mechanism, such as cetuximab or other EGFR-targeted antibodies or through the use of an irreversible inhibitor of EGFR, such as neratinib or afatinib. The combination of afatinib with cetuximab appears to have been the most promising approach in the treatment of patients with acquired resistance to EGFR-TKIs thus far (47).

Amplification of the MET receptor tyrosine kinase was observed in a further 15-20% of patients who underwent EGFR-TKI resistance. This amplification activates downstream intracellular signalling independently of EGFR and seems to occur independently of the T790M mutation (48). MET is a high-affinity tyrosine kinase receptor for hepatocyte growth factor (HGF). Interaction with its ligand has been shown to induce autophosphorylation at multiple tyrosine residues, including PI3K, in an ERBB3-dependent manner, inducing the activation of downstream pathways involved in cell growth, motility, survival, invasion and metastasis (49).

In addition to T790M and *MET* amplification, which are present in the majority of EGFR-TKI-resistant tumours, other phenotypic changes have been shown to be responsible for resistance mechanisms.

In ~10-15% of EGFR-TKI-resistant cases, in the recurrent disease, a diagnosis of small-cell lung cancer (SCLC) was observed in patients, maintaining the original *EGFR* mutations and with the acquisition of neuroendocrine marker expression. These patients were also sensitive to a standard SCLC treatment (38), suggesting that in these cases, characterisation of the specific resistance mechanism can allow for the most appropriate choice of subsequent treatment as well.

Experiments performed in cell lines demonstrated an epithelial-to-mesenchymal transition (EMT) in developing EGFR-TKI resistance, with acquisition and loss of vimentin and E-cadherin expression, respectively. This observation has also been made in cancer patients, where this phenotypic change is associated with a more invasive phenotype (38).

Anti-ALK therapy. Anaplastic lymphoma kinase (ALK) rearrangements were first identified as a fusion to a portion of the nucleophosmin (NPM) gene in 60% of anaplastic large cell lymphomas. ALK rearrangements in NSCLC were identified in late 2007, primarily as fusions to EML4 (50,51). The fusion protein has been identified in ~3-7% of NSCLC patients and is primarily present in lung adenocarcinoma, in young patients and non-smokers or light smokers (50,52). Moreover, this alteration seems to be mutually exclusive with that of EGFR and KRAS. Most of the identified EML4-ALK fusion proteins have been shown to be oncogenic in both *in vitro* and *in vivo* systems (50,53). Pre-clinical and clinical studies have shown that cancer cells harbouring EML4-ALK or other ALK abnormalities are extremely sensitive to ALK inhibitors (54,55). The first clinically available TKI targeting ALK, crizotinib (PF-02341066), showed marked antitumour activity in a phase I study in patients with advanced ALK-positive NSCLC (3). A notable overall 57% response rate and 33% stable disease were observed. These excellent results led to the accelerated approval of the drug in the USA.

Additionally, with regard to crizotinib, several mechanisms of resistance have been demonstrated that occurred following approximately 12 months of treatment.

Acquired resistance to crizotinib was associated with secondary mutations in the *ALK* gene. These mutations either involve the 'gatekeeper' residue (L1196) or sites at a distance from crizotinib binding (F1174L and C1156Y) (56,57). Other mechanisms, such as the activation of HER family signalling, have been demonstrated as ALK-TKI-resistant (58). The cytotoxic activity of crizotinib has also been demonstrated against tumours carrying the *ROS1* rearrangement (4,59) and *MET* amplification (5).

Anti-MET agents. *MET* amplification is a rare *de novo* event in NSCLC patients (60), whereas it is a common mechanism of EGFR-TKI-induced resistance. Numerous target agents have been studied with the intention of inhibiting MET activity and the results for some of these agents are promising.

Tivantinib (ARQ 197) is currently in a phase III trial (marquee) based on a successful randomised phase II study (erlotinib ± tivantinib) (6) in which activity has been demonstrated, particularly in *KRAS*-mutated tumours. MetMAb (Hoffmann-LaRoche, Mississauga, ON, Canada), a monovalent anti-Met monoclonal antibody, has produced significant results in a randomised phase II trial (OAM4558g). In this study, MetMAb, in association with erlotinib, increased PFS and OS significantly compared to erlotinib alone, and the principal predictive factor was the expression of MET evaluated by immunohistochemistry (61). MET is likely to be the next major biomarker in metastatic NSCLC, given the speed with which the different drugs are applied in the clinic.

3. Biological samples suitable for molecular characterisation

Of note for the molecular characterisation of NSCLC patients is that biological material for molecular analyses is not always sufficient or available for inoperable stage IIIB and IV tumours.

The gold standard for molecular analysis is paraffin-embedded histological slides derived from a histological sample, such as a biopsy, where a tumour cell selection has been performed in order to identify a section area with at least 50% of tumour cells. Subsequently, false-negative results may be avoided as non-tumour cells around the tumour may undergo DNA extraction together with tumour cells, leading to contamination of the results.

Approximately one-third of primary NSCLC diagnoses are performed on cytological samples (62-64) and usually no other biopsy materials are available for molecular analyses. Efforts have therefore been focused on detection of *EGFR* mutations in cytological samples and the results of several studies have demonstrated that cytological material is suitable and reliable for *EGFR* mutation analysis (65-70), such as that for the *EML4-ALK* FISH test (68). Specifically, paraffin-embedded histological or cytological sections could, compared to cytological smears, result in truncated cells and nuclei, producing more DNA fragmentation and a consequent possible false number of gene copies.

However, cytological material is usually the only available diagnostic biologic material and it is the only biologic material available in Pathology archives. Its destruction therefore is likely to pose a problem. Moreover, use of only one cytological slide would be insufficient for performing all the necessary molecular analyses.

Authors of the present study as well as other investigators (68-70) have demonstrated that *EGFR* mutation analysis may be performed on a small number of tumour cells (20-30 cells) isolated from a cytological slide, enabling the remaining material to be archived or used for other molecular analyses such as the *EML4-ALK* FISH test.

We have also demonstrated that *EGFR* mutation analysis can be performed subsequent to the *EML4-ALK* FISH test (71). Tumour cells can be scraped off the slide after the FISH analysis and be subjected to DNA extraction and *EGFR* mutation analysis. Thus, the same tumour cells can be used for two different molecular analyses.

The increase in molecular characterisation that has become crucial for the clinical management of patients has led to a search for new approaches to optimise the use of available biological samples. The increasing importance of MET inhibitor agents is likely to require *MET* amplification characterisation, potentially adding to the *EGFR* and *EML4-ALK* analyses that are now required.

A non-invasive approach able to overcome the scarcity of tumour material is the analysis of DNA extracted from plasma/serum or from circulating tumour cells (CTCs). It has been demonstrated that free-tumour-derived DNA levels in plasma or serum are significantly higher in lung cancer patients compared to healthy donors (72,73). This finding may be explained by the presence of necrotic cells sloughed from primary tumour or CTCs, which possess identical genetic lesions.

Kimura *et al* were the first to report the detection of *EGFR* mutations in serum (74,75). In their study, the tumour and serum samples from 42 patients were analysed. *EGFR* mutations were detected in 8 tumour and in 7 serum samples, showing a high concordance between tumour and serum (75). Subsequent studies have attempted to confirm these results in a larger case series (75-89). Using different methodologies, results of the majority of those studies identified identical

Table I. Correlation between *EGFR* mutation status in paired plasma and tumour samples.

Author, year (ref.)	*EGFR*-mutated tumours (n)	Biological material	Methodology	Mutations in paired samples % (n)
Kimura *et al*, 2007 (74)	8	Serum	SARMS	75 (6/8)
Maheswaran *et al*, 2008 (75)	18	Plasma	SARMS	39 (7/18)
		CTC	SARMS	94 (17/18)
Yung *et al*, 2009 (76)	12	Plasma	Digital PCR	92 (11/12)
Kuang *et al*, 2009 (77)	30	Plasma	SARMS and WAVE/Surveyor	70 (21/30)
He *et al*, 2009 (78)	18	Plasma	Mutant-enriched PCR	94.4 (17/18)
Bai *et al*, 2009 (79)	77	Plasma	DHPLC	82 (63/77)
Mack *et al*, 2009 (80)	7	Plasma	SARMS	71 (5/7)
Jiang *et al*, 2011 (83)	18	Serum	Mutant enriched PCR	78 (14/18)
Brevet *et al*, 2011(84)	31	Plasma	Mass spectrometry	61 (19/31)
Taniguchi *et al*, 2011 (85)	44	Plasma	BEAMing	73 (32/44)
Chen *et al*, 2012 (86)	30	Plasma	PNA-LNA PCR	83 (25/30)
Nakamura *et al*, 2012 (87)	39	Plasma	WIP-QP	39 (15/39)
Goto *et al*, 2012 (88)	51	Serum	SARMS	43 (22/51)

CTC, circulating tumoural cells; SARMS, scorpion amplification refractory mutation system; DHPLC, denaturing high-performance liquid chromatography; BEAMing, beads, emulsion, amplification and magnetics; WIP-QP, wild inhibiting polymerase chain reaction and quenched probe system; PNA-LNA PCR, peptide nucleic acid-locked nucleic acid polymerase chain reaction.

serum/plasma, with tissue *EGFR* mutations being reported in >70% of patients (74-89) (Table I).

Moreover, in some of these studies, *EGFR* mutations were found in the plasma but not in the corresponding tumour tissue (80). In their study, Bai *et al* (80) reported that of 77 patients with primary tumour with an *EGFR* mutations, 63 exhibited identical alterations in the matched plasma. Moreover, 7% of patients with plasma mutations had no detectable alterations in the corresponding primary tumours, while 6% of patients with tumour mutations had no detectable *EGFR* alterations in the corresponding plasma. The authors attempted to explain this apparent inconsistency in terms of the heterogeneity of genetic tumour abnormalities, where tumour cells may or may not carry the mutation. A recent study has demonstrated a high discordance rate among multiple pulmonary nodules in terms of *EGFR* mutation, emphasizing the problem of tumour heterogeneity (90) and suggesting that CTC or circulating DNA analyses are crucial in the identification of the presence of mutations and tumour heterogeneity.

The lower tumour cell content in some of the samples may also contribute to the lack of detectable mutations in some tumour tissues in which the corresponding plasma was mutated.

Plasma DNA analysis has also been used to monitor patients during gefitinib treatment, for example, to characterise secondary mutations, such as the T790M alteration (78,86). This may be significant in view of the characterisation of the molecular mechanisms of resistance after TKI treatment in order to select the best subsequent personalised treatment.

The possibility of characterising *EGFR* status in CTCs (76), with high levels of sensitivity has also been demonstrated. However, the technology of CTC enrichment remains to be standardised and generalised, although in recent years efforts have been made to investigate CTC detection and characterisation (91-94).

Nevertheless, the scarcity of materials obtained from the primary tumour tissue of advanced-stage lung cancer patients and from biopsy or cytological samples highlights the potential clinical importance of plasma/serum or CTCs as a surrogate biological sample for genetic analysis.

4. Conclusion

EGFR mutations and *EML4-ALK* translocation analyses are the most clinically relevant alterations that can dictate personalised treatment for NSCLC patients, with EGFR-TKIs or anti-ALK agents, respectively. The growing number of other promising target agents, including MET inhibitors, makes it likely that MET or other markers may aid in decision making. The scarcity of tumour samples available for molecular analysis should be addressed, and different technological approaches may allow for the optimal use of the material, in order to attempt to perform a higher amount of molecular characterisation with a minimal amount of material. Improvement in terms of the sensitivity of molecular biology technologies may therefore prove useful. The possibility of performing molecular characterisations on small amounts of biological material is an important issue, considering the possibility of performing a re-characterisation of tumours following TKI resistance, in order to define the best second-line personalised treatment in view of the specific induced resistance mechanism.

Acknowledgements

The authors would like to thank Professor Rosella Silvestrini for her invaluable scientific contribution and Ursula Elbling for editing the manuscript.

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